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14. ABSTRACT

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Summary

Escherichia coli W3110 was successfully modified to produce D-1,2,4-butanetriol (D-BT) in concentrations exceeding 10 g/L by conversion of D-xylose. The modified strain, *E.coli* WN13, was scaled from 1 L (bench scale) to 100 L (pilot scale) fermentor working volumes at MBI International. D-BT was isolated from fermentation broth by a succession of downstream unit operations, including: filtration, ion exchange, evaporation and distillation. Five-hundred milliliters of D-BT was collected and delivered to the Indian Head Division of the Naval Surface Warfare Center for nitration and testing. "Green" D-BT was nitrated to D-1,2,4-butanetriol trinitrate (D-BTTN) and formulations including this D-BTTN had comparable performance metrics as synthetic D-BTTN. An economic model was constructed to determine the efficacy of the current process and to provide direction for the green production of D-BT.

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LARGE SCALE GREEN SYNTHESIS OF 1,2,4-BUTANETRIOL
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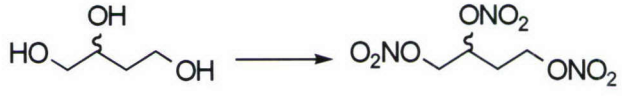
Introduction

A combined research and development effort has investigated the green syntheses of D-BT. The Frost group at MSU has genetically modified *E.coli* for D-BT production and MBI International has developed a process for D-BT production.[3] D,L-1,2,4-butanetriol (D,L-BT) is an intermediate used by the U.S. military for the production of the D,L-1,2,4-butanetriol trinitrate (D-BTTN). Currently, the production costs of generating synthetic D,L-1,2,4-butanetriol make scale-up from the current capacity of 15,000 pounds per year to a desired capacity of 180,000 pounds per year economically infeasible. MSU and MBI have proposed an alternative route that is potentially low cost and relatively environmentally benign. The aim of this project was to assess the alternative green synthesis of D-BT for the subsequent production of D-BTTN.

Nitration of racemic BT yields BTTN (Scheme 1), which is an energetic nitrate ester used in propellants and explosives. Racemic BTTN is used as an energetic plasticizer for both extrudable and castable minimum smoke propellant compositions and as a coplasticizer in castable explosives formulations. The applications and use of BTTN are similar to those of nitroglycerin (NG). However, substitution of BTTN for NG in plasticizer applications would reduce hazards associated with the propellant manufacturing process and hazards associated with use of the final product. The expense of racemic BT ($30-$40/lb) currently limits its use as a precursor to BTTN to approximately 15,000 lb/yr.[2b] If the cost of BT could be reduced ($10-15/lb), future use of BT is projected to increase to 180,000 lb/yr.[2b]

Scheme 1



1,2,4-butanetriol
BT

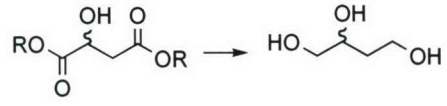
1,2,4-butanetriol trinitrate
BTTN

Because only racemic BTTN is used in high energy material applications, racemic D,L-BT has been exclusively employed as the precursor to this nitrate ester. Preliminary results indicate that the physical properties of L-BTTN are nearly identical to D,L-BTTN when analyzed by differential scanning calorimetry and thermomechanical analysis.[4] This indicates that D-BT, L-BT, and mixtures of these enantiomers are suitable substrates for nitration.

Chemical reduction of racemic malic acid constitutes the most direct route to racemic BT. Malic acid is commercially produced by the acid-catalyzed hydration of fumaric acid, which in turn, is obtained from maleic anhydride.[5] Malic acid (Scheme 2) has been stoichiometrically reduced to BT in quantitative yield using borane-dimethyl sulfide in tetrahydrofuran.[6a] Commercially, BT is manufactured by the reduction of dimethyl malate (Scheme 2) using $NaBH_4$ in a mixture of a $C_{2\text{-}6}$-alcohol and tetrahydrofuran.[6b,c] As might be expected for a stoichiometric reduction, a byproduct salt stream is generated. Each ton of dimethyl malate reduced using $NaBH_4$ results in the generation of 2-5 tons of borate salts.[6b,c] Byproduct salt streams combined with the expense

of the stoichiometric reductant are major impediments to use of this reaction at a scale where the availability of product BT would be comparable to current availability of *glycerol*.

Scheme 2



R=H malic acid
R=CH_3 dimethyl malate

1,2,4-butanetriol

As for catalytic reductions, hydrogenation of esterified D,L-malate using copper chromite catalysts has been used but suffers from the toxicity of Cr^{+6}.[7] More recently, hydrogenation of aqueous solutions of malic acid has been examined using 5% Ru on C.[3,8] High pressures of H_2 are required when either Ru or $2CuO{\cdot}Cr_2O_3$ are employed as catalysts.[3,7,8] The best results using Ru on C are realized when malic acid is hydrogenated at 5,000 psi and 135 °C.[3,8] In addition to a 74% yield of BT, 24% of the starting malic acid is converted to a mixture of 1,2-propanediol, 1,4-butanediol, ethylene glycol, and 1,2-butanediol.[3,8] Beyond yield reduction, byproduct formation introduces contaminants that are difficult to separate from BT by distillation.[3,8] The optimized hydrogenation requires 1.3 mol% of Ru relative to malic acid.[3,8] The expense of Ru and the quantity of this metal required for large-scale hydrogenation of malic acid would constitute a significant cost factor.

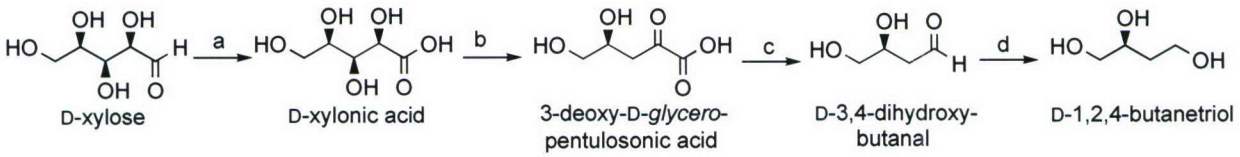
In contrast to $NaBH_4$ reduction and Ru-catalyzed hydrogenation of malic acid, the microbes required for the pentose-based routes to BT only need to be prepared (constructed) a single time. Their ability to rapidly grow from a single cell essentially means that microbial catalysts "prepare" themselves. Though salts are required for growth of microbes, the quantities and concentrations of the inorganic phosphate, ammonium, and sulfate salts required for microbial growth and metabolism are small relative to the byproduct borate salts produced during a stoichiometric reduction of dimethyl malate.[6b,c] The microbe-catalyzed syntheses can also be run at atmospheric pressures and temperatures that do not exceed 37 °C. These are much milder reaction conditions relative to the 5,000 psi H_2 pressures and 135 °C reaction temperatures required for catalytic hydrogenation of malic acid.[3,8]

For a cost-effective green synthesis of BT, an inexpensive source of D-xylose and L-arabinose starting materials is needed. These pentoses can be obtained from the fiber fraction isolated from corn hulls during milling of corn. Up to 50% by weight of corn hulls is a highly-branched heteroxylan composed mostly of D-xylose and L-arabinose.[9] The backbone of the heteroxylan consists of β-(1,4)-linked xylose residues. Approximately 80% of the backbone xylose residues are substituted at *O*-2 or *O*-3 by monomeric and oligomeric side chains.[9] L-arabinose is a major component of these side chains. Heteroxylan depolymerization can be achieved using dilute sulfuric acid at elevated temperatures[10] or hemicellulases/xylanases.

One method for the green synthesis of D-BT (Scheme 3) involves a two-microbe process. The first organism, *P. fragi* ATCC4973, oxidizes D-xylose to D-xylonic acid. The second organism,

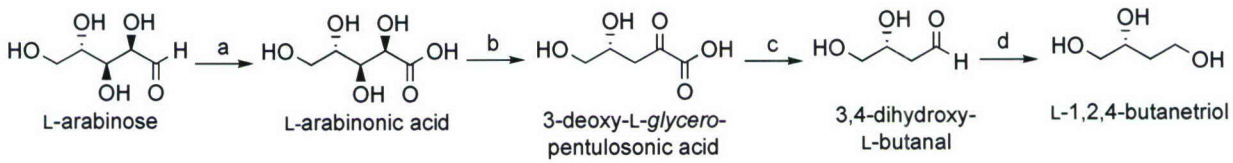
recombinant *E.coli* DH5α/pWN6.186A, converts D-xylonic acid to D-BT. A two-microbe process can also be used for the synthesis of L-BT. L-Arabinose is oxidized by *P. fragi* ATCC4973 and the resulting L-arabinonic acid can be converted into L-BT by recombinant *E.coli* BL21(DE3)/pWN6.222A (Scheme 4). Importantly, the same strain of *P. fragi* can oxidize both D-xylose and L-arabinose. The disadvantage of this approach concerns the purification of the D-xylonic acid between the two fermentations. Additional processing steps, between these two fermentations, add to capital and processing costs. The use of a single organism for the direct conversion of D-xylose to D-BT has the potential to reduce costs by eliminating the need for mid-stream processing. MBI International and MSU have developed a single-microbe process that produces high-purity D-BT. The chromosomal DNA of *E.coli* W3110 was modified to encode for D-xylose dehydrogenase, the first enzyme needed for xylose conversion (Scheme 32). This organism was further transformed by plasmid DNA containing the mdLC gene encoding for benzoylformate decarboxylase. When expressed, benzoylformate decarboxylase converts 3-deoxy-D-*glycero*-pentulosonic acid to 3,4-dihydroxybutanal. These modifications coupled with the organisms native ability to dehydrate aldonic acids (via D-xylonate dehydratase) and convert aldehydes to alcohols (via alcohol dehydrogenase) results in *E.coli* WN13/pWN7.126B.

Scheme 3



a. D-xylose dehydrogenase; b. D-xylonate dehydratase;
c. 2-ketoacid decarboxylase; d. alcohol dehydrogenase

Scheme 4



a. L-arabinose dehydrogenase; b. L-arabinonate dehydratase;
c. 2-ketoacid decarboxylase; d. alcohol dehydrogenase

In addition to raw materials cost, the cost of downstream processing is often significant in bioprocesses that use fermentations for conversion. The development of a sequence of unit operations for the economical separation D-BT from the fermentation broth is necessary prior to scale up to the process level. Several technologies can be used to generate an isolation scheme for D-BT purification. A solid/liquid separation in the form of centrifugation or membrane filtration is often employed to remove microorganisms and other solids from the broth. Ions and proteins can be removed using ion exchange chromatography. Water removal is typically

accomplished by evaporation. D-BT can be purified from the remaining impurities by distillation in a short-path molecular still. D-BT may also be removed from fermentation broth using chemical extraction of a derivatized form of D-BT. Aldehydes such as benzaldehyde are candidates for D-BT derivatization to add a hydrophobic moiety. The derivatized D-BT may be extracted by excess amounts of the aldehyde itself. Product isolation follows by distillation of the aldehyde and the stoichiometric addition of water to hydrolyze the aldehyde moiety from D-BT. As other isolation processes can be envisioned, the costs associated with the various isolation processes are the metrics for deciding which process is best for scale up.

Approach

General approach

The first reactive step in the synthesis of D-1,2,4-butanetriol is the conversion of D-xylose to D-xylonic acid. *P. fragi* is known to convert both D-xylose to D-xylonic acid and L-arabinonic acid. Aldonic acids from generated by *P. fragi* using corn fiber-derived pentose sugars. *E.coli* was transformed to impart chromosomal expression for the conversion of D-xylose to D-xylonic acid. The subsequent conversion of aldonic acids to D,L-pentulosonic acid is native to *E.coli* W3110. Converting aldonic acids to 3,4-dihydroxybutanal was accomplished by transforming *E.coli* via a plasmid containing benzoylformate decarboxylase. Alcohol dehydrogenase catalyzes the final step in the conversion. The transformed organism was grown at 1 L, 10 L and 100 L scales. A potentially scalable downstream process was developed for separating D-BT from fermentation broth. An economic model was constructed to evaluate D-BT production and guide future research and development.

Organism improvement

Benzoylformate decarboxylase and pyruvate decarboxylase were identified for conversion of 3-deoxy-D-*glycero*-pentulosonic acid. Benzoylformate decarboxylase from *Pseudomonas putida* and pyruvate decarboxylase from *Zymomonas mobilis* were subjected to site-directed mutagenesis. These mutant isozymes were then analyzed to identify increased reactivity towards 3-deoxy-D,L-*glycero*-pentulosonic acid. In parallel, an informatics approach was employed to identify genes in genome databases that possessed a significant amount of sequence homology with benzoylformate decarboxylase from *P. putida*. Microbes possessing these tentatively assigned benzoylformate decarboxylase homologues were obtained and the relevant genes isolated. Amplified expression of these homologues in *Eschericha coli* was followed by analyses to identify activity with respect to decarboxylation of 3-deoxy-D,L-*glycero*-pentulosonic acid. As only *P. putida* benzoylformate decarboxylase catalyzed the conversion of 3-deoxy-D-*glycero*-pentulosonic acid, plasmids with the mdlC inserts encoding benzoylformate decarboxylase were transformed into *E.coli* W3110. This strain was evaluated for the synthesis of D-1,2,4-butanetriol from D-xylonic acid under fermentor-controlled conditions. The impact of competition for 3-deoxy-D-*glycero*-pentulosonic acid was then explored with the construction of an *E.coli* W3110ΔyjhHΔyagE double knockout, which was incapable of converting 3-deoxy-D-*glycero*-pentulosonic acid into pyruvate and glycolaldehyde. The specific activities of native D-xylonic acid dehydratase and benzoylformate decarboxylase were also followed over time.

Based on the low concentrations of synthesized D-1,2,4-butanetriol, constitutive overexpression of benzoylformate decarboxylase was explored.

Continued research has focused on improving the benzoylformate decarboxylase expression level. *mdlC* encoding benzoylformate decarboxylase has been cloned into a phage T7 based expression vector pET28C(+) to yield plasmid pML7.135. Two other recombinant plasmids have been constructed co-expressing *E.coli* chaperonin *groEL-groES* and *mdlC* genes. DE3 lysogen host strain KIT15 has been prepared for protein expression under a phage T7 promoter. KIT15/pML7.135 was proven to be a 3-deoxy-D-*glycero*-pentulosonic acid producing biocatalyst with a titer of 23 g/L under fermentor-controlled conditions.

Upstream processing
Corn fiber was pretreated by AFEX and the resultant glucan was enzymatically converted to monosaccharide. Saccharification of the cellulose fraction results in the solubilization of hemicellulose. Solid/liquid separation was followed by the precipitation of hemicellulose from the supernantant using ethanol. Pentose sugars were generated by acid hydrolysis of the hemicellulose solid (a.k.a. corn fiber gum). This stream was neutralized using calcium hydroxide and the resultant gypsum was removed. The fermentability of the pentose-rich supernatant was assessed using *P. fragi*. The resultant aldonic acids, D-xylonic acid and L-arabinonic acid, were quantified using high-pressure liquid chromatography with refractive index detection.

Fermentation
In the second year of the grant, *P. fragi*-generated D-xylonic acid was converted to D-1,2,4-butanetriol by recombinant *E.coli*. *E.coli* fermentations were performed at the 2.5 L and 10 L scales. The downstream isolation of D-1,2,4-butanetriol was accomplished by a sequence of centrifugation, precipitation, filtration, and evaporation. Specifically—*E.coli* were removed by centrifugation; water was removed by evaporation; methanol was used to remove oligosaccharides, proteins, and DNA; methanol and water were removed by evaporation; and the D-1,2,4-butanetriol was distilled in a short-path molecular still.

In the third year of the grant, a new *E.coli* construct, WN13, was completed by MSU that was capable of fermenting D-xylose directly to D-1,2,4-butanetriol. The fermentation was scaled to 100L and run three times at this scale. This eliminated the need for the separate production and isolation of xylonic acid.

Downstream processing
The use of specialized equipment (specifically a Karr column) and a reactive extraction method were both explored for their utility in recovering the completely water-soluble D-1,2,4-butanetriol from an aqueous process stream. The final downstream isolation of the D-1,2,4-butanetriol was achieved via membrane filtration, the use of ion-exchange resins to remove the remaining charged components of the process stream (both organic and inorganic), removal of water by evaporation under reduced pressure, and purification of the crude D-1,2,4-butanetriol product by short-path distillation. All these process steps are amenable to industrial scale-up.

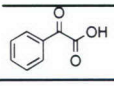
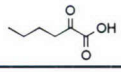
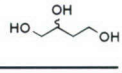
Results and Discussion

Organism improvement

In unpublished work, MacLeish and coworkers have manipulated amino acid residues in benzoylformate decarboxylase from *Pseudomonas putida* and pyruvate decarboxylase from *Zymomonas mobilis* to significantly alter the specificity of these enzymes. These site-directed mutations were based on analysis of the crystal structures of these enzymes. We made a series of mutations based on the amino acid substitution discovered by MacLeish to lead to significant alterations in substrate specificity. Mutants were constructed using Pfu turbo DNA polymerase and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Genes encoding the mutant isozymes of benzoylformate decarboxylase and pyruvate decarboxylase were all localized behind a Plac promoter in plasmids, which also carried a lacIQ insert. Decarboxylase activities were measured in crude cell lysate using a coupled enzyme assay based on intermediacy of decarboxylase-produced benzaldehyde, pentanal, or D,L-3,4-dihydroxybutanal. Reduction of these intermediates by horse liver alcohol dehydrogenase and concomitant oxidation of NADH was measured by the loss of absorbance at OD 340 nm. A second analysis was also employed for detecting formation of D,L-1,2,4 butanetriol from 3-deoxy-D,L-*glycero*-pentulosonic acid. Concentrated product mixtures were derivatized using bis(trimethylsilyl)trifluoroacetamide (BSTFA) and the yield of persilylated product D,L-1,2,4-butanetriol determined by GC relative to added dodecane standard.

The I472A mutation (pML3.062) was previously identified by McLeish and coworkers to rather dramatically change the specificity of pyruvate decarboxylase. Whereas wild-type pyruvate decarboxylase (pLO1276) displayed little activity towards either benzoylformate or hexulosonate, the I472A mutant (pML3.062) showed a noteworthy increase in activity towards both of these substrates. Unfortunately, the I472A mutant (pML3.062) did not display significant activity when 3-deoxy-D,L-*glycero*-pentulosonic acid was employed as a substrate. Other variants such as a I472S mutant (pML3.084), a I472G mutant (pML3.086), and a I472T mutant also displayed insignificant decarboxylase activity relative to 3-deoxy-D,L-*glycero*-pentulosonic acid. In each of these instances, trace levels of persilylated D,L-1,2,4-butanetriol product was detected by the GC analysis (Table 1).

Table 1. Specific Activities and Product D,L-1,2,4-Butanetriol Yields Resulting from Site-Directed Mutagenesis of *mdlC*-encoded Benzoylformate Decarboxyase and *pdc*-encoded Pyruvate Decarboxylase.

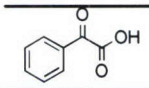
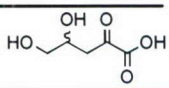
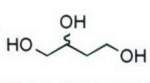
Plasmid	Origin	Mutation	Enzyme Activity (U/mg)[1]			Yield (%)[2]
						
pWN5.238A	*P. putida*	wild-type	26.2	0.2	0.01	5.3
pML3.054	*P. putida*	A460I	0.5	0.3	<0.01	0.2
pLOI276	*Z. mobilis*	wild-type	0.03	0.08	<0.01	0
pML3.062	*Z. mobilis*	I472A	0.3	10.3	<0.01	0.03
pML3.084	*Z. mobilis*	I472S	<0.01	0.13	<0.01	0.08
pML3.086	*Z. mobilis*	I472G	0.01	1.26	<0.01	0.04
pML3.104	*Z. mobilis*	I472T	<0.01	0.14	<0.01	0.04

[1] units (U) = 1 μmol/min. [2] mol/mol

The BLAST sequence alignment algorithm was used to search the NCBI database for sequences bearing significant sequence identity with the *P. putida* mdlC gene encoding benzoylformate decarboxylase. Eight microbes were identified to carry a gene having significant sequence identity to mdlC from *P. putida*. Genomic DNA was extracted from each of these microbes and Pfu turbo DNA polymerase was used to amplify the putative decarboxylase genes. These genes were subsequently cloned into plasmid pJF118EH. Inserts in this vector were under Ptac promoter control. Plasmid pJF118EH also carried a lacIQ insert.

Crude cell lysates were analyzed using a coupled enzyme assay and determination of the yield of biosynthesized D,L-1,2,4-butanetriol as previously described for the site-directed mutagenesis experiments. The only significant activity in the coupled enzyme assay was observed for *E.coli* BL21/pML3.040 (Table 2), which expressed the *Acidithiobacillus ferridoxidans* benzoylformate decarboxylase homolog. Due to high background dehydrogenase activity in the crude cell lysates, the measured yields of D,L-1,2,4-butanetriol proved to be a more reliable measure of activity with respect to decarboxylation of 3-deoxy-D,L-*glycero*-pentulosonic acid. Low, albeit significant yields of D,L-1,2,4-butanetriol were observed for five of the heterologously expressed benzoylformate decarboxylase homologs (Table 2).

Table 2. Specific Activities and Product D,L-1,2,4-Butanetriol Yields Resulting from Heterologous Expression of Benzoylformate Decarboxylate from Various Microbial Sources.

Construct	Bacteria	% Identity[1]	Enzyme Activity (U/mg)[2] 		Yield (%)[3] 
DH5α/pWN5.238A	*Pseudomonas putida*		25.5	0.01	5.3
BL21/pML2.118	*Pseudomonas fluorescens*	60	0.79	0.003	0.5
BL21/pML2.123	*Pseudomonas aeruginosa*	59	0.26	<0.01	0.6
BL21/pML2.162	*Burkholderia fungorum*	61	0.51	<0.01	0.15
BL21/pML3.040	*Acidithiobacillus ferrooxidans*	37	0.01	0.03	0.5
BL21/pML2.208	*Streptomyces coelicolor*	49	0.03	<0.01	<0.01
BL21/pML2.214	*Novosphingobium aromaticivorans*	37	0.003	<0.01	<0.01
BL21/pML2.256	*Rhodopseudomonas palustris*	40	0.005	<0.01	<0.01
BL21/pML2.286	*Mycobacterium smegmatis*	42	0.006	<0.01	0

[1] sequence identity with mdlC-encoded benzoylformate decarboxylase from *P. putida*; [2] units (U) = 1 μmol/min; [3] mol/mol.

The first microbial construct, *E.coli*DH5α/pWN6.186A, reported to be capable of synthesizing D-1,2,4-butanetriol from D-xylonic acid required nutritional supplementation. An *E.coli* W3110 construct was therefore pursued, which would be capable of growth and biosynthesis of D-1,2,4-butanetriol in unsupplemented minimal salts medium under fermentor-controlled conditions. In route to this goal, plasmid pWN7.126B was constructed, which carried a mdlC insert encoding benzoylformate decarboxylase from *Pseudomonas putida*. The mdlC insert was expressed from a Ptac promoter. Plasmid pWN6.186A also carried a lacIQ insert, which required addition of IPTG to the culture medium of *E.coli* W3110serA/pWN7.126B for expression of benzoylformate decarboxylase.

E.coli W3110serA/pWN7.126B was cultured under fermentor-controlled conditions in minimal salts medium using glucose as the sole carbon source at 33 °C with pH 7.0 maintained throughout the fermentation. Three staged methods were used to maintain D.O. concentrations at 10%. With the airflow at an initial setting of 0.06 L/L/min, the D.O. concentration was maintained by increasing the impeller speed from its initial set point of 50 rpm to 940 rpm. With the impeller speed constant, the mass flow controller then maintained the D.O. concentration by increasing the airflow rate from 0.06 L/L/min to a preset maximum of 1.0 L/L/min. At constant impeller speed and constant airflow rate, the D.O. concentration was finally maintained at the desired air saturation for the remainder of the cultivation by oxygen sensor-controlled glucose feeding. At the point when glucose feeding started, IPTG stock solution was added into the fermentor to a final concentration of 0.5 mM. D-Xylonic acid was then added to initiate the biosynthesis of D-1,2,4-butanetriol. Under these conditions, 0.08 g/L of D-1,2,4-butanetriol was synthesized by *E.coli* W3110serA/pWN7.126B.

Attention turned to eliminating the conversion of intermediate 3-deoxy-D-*glycero*-pentulosonic acid into pyruvate and glycolaldehyde. This reaction is catalyzed by two isozymes of 3-deoxy-D-*glycero*-pentulosonate aldolase encoded by the yjhH and yagE loci in *E.coli*. These loci were site specifically inactivated in *E.coli* W3110ΔyjhHΔyagEserA. Cultivation of *E.coli* W3110ΔyjhHΔyagEserA/pWN7.126B under the aforementioned fermentor-controlled conditions resulted in the synthesis of 8.3 g/L of D-1,2,4-butanetriol in 45% yield from D-xylonic acid. Further analysis of the culture supernatant of *E.coli* W3110ΔyjhHΔyagEserA/pWN7.126B revealed the formation of 5.6 g/L of 3-deoxy-D-*glycero*-pentanoic acid. Formation of D-1,2,4-butanetriol and 3-deoxy-D-*glycero*-pentanoic acid accounted for 66% (mol/mol) of the D-xylonic acid added to the culture medium. It thus appeared that dehydrogenase activity was now competing with benzoylformate decarboxylase for 3-deoxy-D-*glycero*-pentulosonic acid.

The specific activities of D-xylonate dehydratase and benzoylformate decarboxylase were also monitored (Figure 1). D-Xylonate dehydratase and benzoylformate decarboxylase catalyze the first and second reactions, respectively, in the microbial synthesis of D-1,2,4-butanetriol from D-xylonic acid. Expression of D-xylonate dehydratase relies on expression of the yagF and yjhG loci in the *E.coli* genome. Benzoylformate decarboxylase was encoded by the mdlC insert in pWN7.126B under the control of a Ptac promoter. With the lacIQ insert also included on pWN7.126B, addition of IPTG was required to induce expression of benzoylformate decarboxylase.

Following the addition of D-xylonic acid under the glucose limited culture conditions, D-xylonate dehydratase expression was induced immediately (Figure 1). The specific activity of this enzyme reached a maximum value and then steadily decreased as D-xylonic acid was depleted in the culture medium (Figure 1). The specific activity of D-xylonate dehydratase then reverted to a basal level. After the addition of IPTG, the specific activity of benzoylformate decarboxylase increased steadily (Figure 1). However, when the specific activity of D-xylonate dehydratase reached its maximum value, which is also the time point of complete D-xylonic acid consumption, the specific activities of benzoylformate decarboxylase were much lower than the maximum values ultimately attained at longer time points (Figure 1).

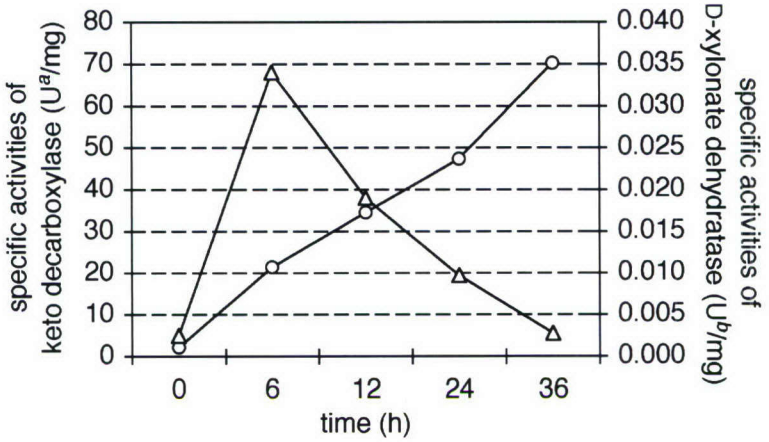


Figure 1. Benzoylformate decarboxylase (open circles) and D-xylonate dehydratase (open triangles) specific activities of *E. coli* JWF1/pWN7.126B cultured under fermentor-controlled conditions. [a] One unit (U) of benzoylformate decarboxylase corresponds to the formation of 1 μmol of benzaldehyde per min at 25 °C. [b] One unit (U) of D-xylonate dehydratase corresponds to the formation of 1 μmol of 3-deoxy-D-*glycero*-pentulosonic acid per min at 37 °C.

In attempt to address this mismatch between the expression levels of D-xylonate dehydratase and benzoylformate decarboxylase, plasmid pWN8.106B was constructed where the plasmid lacIQ insert was inactivated. Surprisingly, constitutive expression of mdlC-encoded benzoylformate decarboxylase in *E.coli* W3110ΔyjhHΔyagEserA/pWN8.106B resulted in a complete absence of D-1,2,4-butanetriol formation. Apparently, constitutive expression of benzoylformate decarboxylase is too great of a metabolic burden on the cell.

E.coli WN13 was derived from *E.coli* WN7, which is a W3110 strain with a *ΔyjhHΔyagEserA* phenotype. YjhH and YagE are two isozymes of 3-deoxy-D-*glycero*-pentulosonate aldolase. Conversion of 3-deoxy-D-*glycero*-pentulosonic acid to pyruvic acid and glycoldehyde catalyzed by YjhH and YagE is a key step in the catabolism of D-xylonic acid by *E.coli* W3110. YjhH and YagE aldolase activities compete with *mdlC*-encoded benzoylformate decarboxylase for 3-deoxy-D-*glycero*-pentulosonic acid. MdlC-catalyzed onversion of 3-deoxy-D-*glycero*-pentulosonic acid to 3,4-dihydroxy-D-butanal is one of the steps required for microbial biosynthesis of D-1,2,4-butanetriol from D-xylonic acid. As demonstrated in Year 2, higher concentrations and yields of D-1,2,4-butanetriol can be synthesized from D-xylonic acid in constructs possessing *ΔyjhHΔyag* deletions.

E.coli WN13 was derived by replacing the genomic *xylAxylB* cluster in WN7 with a *xdh*(*C. crescentus*)-Cm^R gene cassette. The *xylA* gene encodes D-xylose isomerase while *xylB* encodes D-xylulose kinase. These are two enzymes essential for *E.coli* catabolism of D-xylose. The *xdh* gene encodes xylose dehydrogenase and was isolated from *Caulobacter crescentus* CB15. Xdh catalyzes the oxidation of D-xylose to D-xylonic acid using NAD as the cofacter. Cm^R encoded

resistance to chloramphenicol. The chromosomal modification of WN7 resulted in the inability of WN13 to utilize D-xylose as a sole carbon source for growth. A second consequence of the chromosomal modification of WN7, is that WN13 expresses *xdh*-encoded D-xylose dehydrogenase activity from the native *xylA* promoter.

The DNA fragment used for chromosomal replacement of *xylAxylB* inWN7 was amplified from plasmid pWN9.068A using the following primers: 5'-TACGACATCATCCATCACCCGCGGCATTACCTGATTATGTCCTCAGCCAT CTATCCC and 5'-CAGAAGTTGCTGATAGAGGCGACGGAACGTTTCTCATATGAATAT CCTCCTTAGT. Plasmid pWN9.068A carries the *xdh* gene adjacent to Cm^R that is flanked by FLP recognition (FRT) sites. PCR fragments were digested with a restriction enzyme and purified by electrophoresis. The purified DNA fragments were introduced into *E.coli* W3110/pKD46 by electroporation. Plasmid pKD46 encodes the phage l red homologous recombinase. Candidates of strain W3110*xylAB*::*xdh*-Cm^R were selected on LB medium containing chloramphenicol. *E.coli* WN13 was generated by P1 phage-mediated transduction of *xylAB*::*xdh*-Cm^R into the genome of WN7. The resulting WN13 was then transformed to afford *E.coli* WN13/ pWN7.126B. Plasmid pWN7.126B carried *serA* and a *mdlC* insert expressed from a P_{tac} promoter.

Attempts to optimize the extraction or derivatization/extraction of D-1,2,4-butanetriol from fermentation broth were compromised by the concentrations of 3-deoxy-D-*glycero*-pentulosonic acid and 4,5-dihydroxy-*erythro*-L-norvaline. By increasing the activity of *mdlC*-encoding benzoylformate decarboxylase, the concentration of the aforementioned carboxylic acids might be reduced or entirely eliminated.

A 1.6 kb DNA fragment encoding *P. putida* benzoylformate decarboxylase gene *mdlC* and a 1.6 kb *serA* locus were sequentially cloned into pET28C(+) to afford plasmid pML7.135. The DE3 lysogen of *E.coli* KIT2 was prepared using the DE3 lysogenation kit of Novagen according to the manufacturer's protocol. The resulting host strain *E.coli* KIT15 was subsequently transformed with pML7.135 to afford biocatalyst KIT15/pML7.135. This biocatalyst was fermented under glucose-limited fed-batch conditions with D-xylose as substrate and evaluated for the production of D-1,2,4-butanetriol along with other organic acid side-products such as D-xylonic acid, 3-deoxy-D-*glycero*-pentulosonic acid, 3-deoxy-D-*glycero*-pentanoic acid, 4,5-dihydroxy-*threo*-L-norvaline, and 3,4-dihydroxy-D-butanoic acid. Comparing with WN13/pWN7.126B, which produced a titer of 10.5 g/L D-1,2,4-butanetriol , KIT15/pML7.135 produced only 1.7 g/L. Interestingly, this construct yielded 23 g/L 3-deoxy-D-*glycero*-pentulosonic acid, which accounted to 80% of the total starting D-xylose in the system.

Despite the failed attempt to increase benzoylformate decarboxylase activity using a phage T7 expression vector, we created a promising 3-deoxy-D-*glycero*-pentulosonic acid biocatalyst by accident. In order to gain a better understanding of the above discovery, biocatalyst KIT15/pRC1.55B was constructed. Plasmid pRC1.55B carries the *serA* gene locus in order to complement the *serA* mutation in *E.coli* KIT15. The lack of *mdlC* encoding benzoylformate decarboxylase in this new biocatalyst should in theory give a similar titer of 3-deoxy-D-*glycero*-

pentulosonic acid as obtained in KIT15/pML7.135. The cultivation of this new construct, however, yielded only 11.8 g/L 3-deoxy-D-*glycero*-pentulosonic acid. The fact that nearly half of the D-xylose was recovered in the broth indicated that the T7 promoter is not suitable for producing functional decarboxylase, presumably due to protein misfolding. On the other hand, however, transforming pML7.135 into *E.coli* KIT15 does promote D-xylose uptake into the cell by an unknown machinery to produce 3-deoxy-D-*glycero*-pentulosonic acid as the major product.

Another attempt to increase benzoylformate decarboxylase activity was done by co-expressing *E.coli* GroEL-GroES chaperone team together with MdlC. As a first step, the plasmid pML7.166 with the *groEL-groES* gene fragment inserted after a *E.coli* P_{tac} promoter was constructed. The 1.5 kb P_{tac}-*groEL-groES* locus was amplified by PCR and subsequently cloned into two *mdlC* expressing plasmids, pWN7.126B (*mdlC* under a P_{tac} promoter) and pML7.135 (*mdlC* under a phage T7 promoter), respectively. The newly constructed plasmids pML7.180 and pML7.202 were then transformed into their suitable *E.coli* host strains to afford biocatalysts KIT2/pML7.180 and KIT15/pML7.202, and evaluated under fermentor-controlled conditions. These new biocatalysts produced no detectable amount of D-1,2,4-butanetriol, but accumulated 21 g/L and 20 g/L 3-deoxy-D-*glycero*-pentulosonic acid, respectively. The clarified broth obtained from these fermentations appeared to have a more complicated composition, as indicated by ^{1}H NMR, due to the higher concentration of other organic acid side-products as compared to KIT15/pML7.135.

Process development

The optimum corn fiber pretreatment conditions using the Ammonia Fiber Expansion (AFEX) process were known. It was also known that enzymatic hydrolysis of AFEX-treated corn fiber with industrial cellulase, cellobiase, and amylase digested approximately 80% of the cellulose plus starch while only digesting approximately 10% of the xylan and arabinan. Corn fiber hemicellulose is soluble in water over a wide range of pH's. It was hypothesized the digestion of the cellulose and starch matrix would solubilize the hemicellulose into solution. After removing the residual solids from the hydrolyzate solution, solubilized hemicellulose and monosaccharides were measured in solution, where the liquid contained 76% of the xylan, 64% of the galactan, and 70% of the arabinan from the starting corn fiber material.

Hemicellulose can be removed from aqueous solution by precipitation with ethanol. Analysis of recovered hemicellulose revealed that all solubilized hemicellulose was precipitated. The resulting hemicellulose was 62% polysachharide by mass. The component polysaccharide content of the hemicellulose was 35% xylan, 18% arabinan, 6% galactan, and 3% glucan. The remaining 38% of the mass is currently unknown. The component mass balance around the extraction process is summarized in Figure 2.

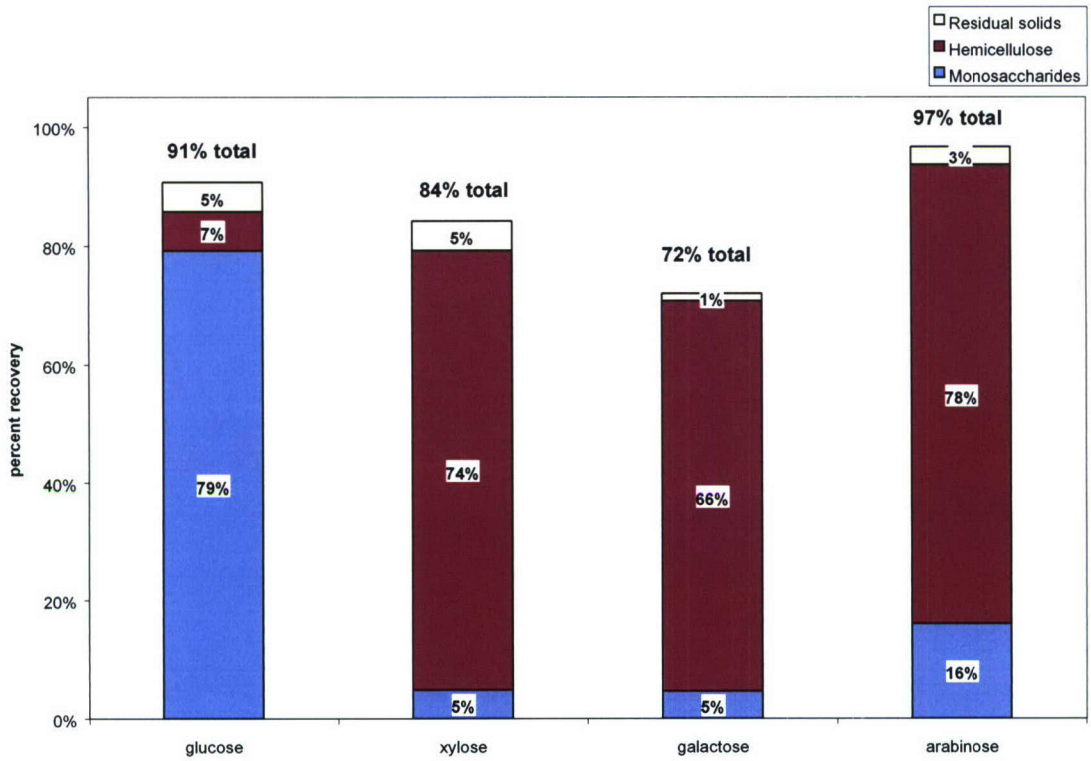


Figure 2. Mass balance around hemicellulose extraction process.

An initial, qualitative investigation of acid hydrolysis of extracted corn fiber hemicellulose was performed. Recovered hemicellulose was dissolved in solution at 10 wt% and hydrolyzed at 50 °C for 24 hours and 121 °C for 1 and 2 hours. Each condition was treated with 1 and 2% sulfuric acid. At 50 °C, only a small amount of the hemicellulose was converted to monosaccharides. At 121 ° C, substantially all the hemicellulose was hydrolyzed.

Hydrolysate, diluted to 80% of the parent concentration, was fermentable by *P. fragi* to aldonic acids—though the run time of 143 hours is excessively long. Hydrolysate that was diluted to 60% of the parent hydrolysate concentration was deemed the optimum case, as the run time of 52 hours produced D-xylonic acid titers of 15 g/L.

The fermentation of D-xylonic acid by *E.coli* resulted in D-1,2,4-butanetriol titers approaching 10 g/L in 60 hours of operation. During the course of the fermentation, the working volume increased 40% (due to acid, base and glucose addition) and the optical density increased to 70 (at 600 nm). The dissolved oxygen level was controlled by a PI (proportional-integral) controller. Maintaining a glucose concentration lower than 0.5 mM was found to be necessary for converting D-xylonic acid to D-1,2,4-butanetriol.

Downstream processing consisted of a sequence of centrifugation, precipitation, filtration and evaporation (Figure 3). The centrifuge was operated at 9500 g for 20 minutes to produce a cell

pellet. Water was removed from the supernatant using a Buchi rotovap at 70 °C and a vacuum pressure of 26 inHg. Methanol was added to the rotovap residue, in a 3:1 volume to volume ratio, to precipitate DNA, oligosaccharides and proteins. Filtration, using a Whatman GF/D filter, was used to separate the solids from the D-1,2,4-butanetriol containing filtrate. Removing the solids at this stage benefits the subsequent unit operations by reducing the amount of particulate matter that appears upon concentration. The Buchi rotovap removed methanol and water to achieve a BT enriched solution with a viscosity adequate for processing in the short-path molecular still. The second rotovap residue was fed into the short-path molecular still at a rate of 60 ml/hr. The still was operated at 167 °C and 5 mmHg, conditions which were found to be capable of volatilizing BT. The recovered distillate was 65% (w/w) D-1,2,4-butanetriol, with the remainder likely being water. The presence of D-1,2,4-butanetriol was confirmed using 1H-NMR, 13C-NMR, and derivatization followed by GC with flame ionization detection.

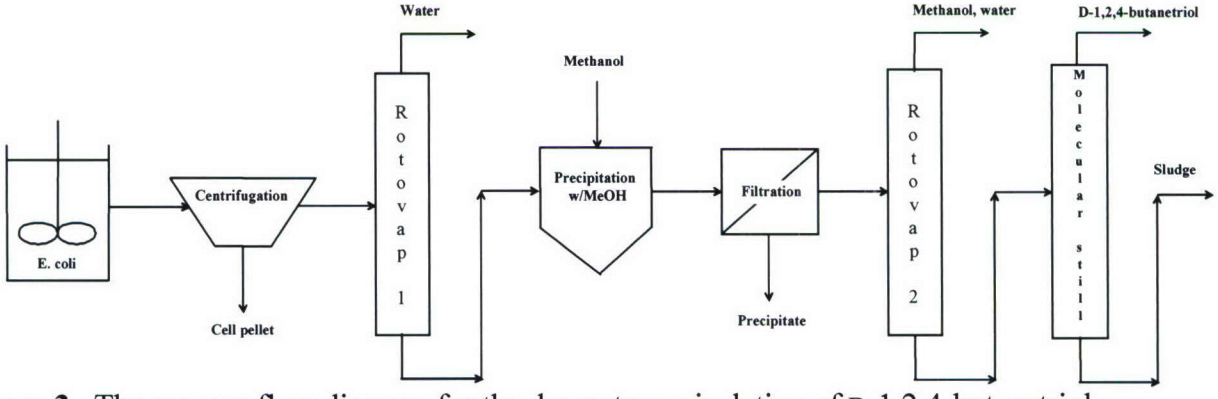


Figure 3. The process flow diagram for the downstream isolation of D-1,2,4-butanetriol. Further effort is needed to isolate D-1,2,4-butanetriol in a cost-effective manner.

The fermentation of D-xylose by *E coli* WN13/pWN7.126B was successfully scaled from 2L, to 10L, to 100L, with D-1,2,4-butanetriol titers of 10 g/L in approximately 60 hours of operation at 100L. During the course of the fermentation, the working volume increased 40% through acid and base addition for pH control, glucose feeding, and xylose addition. During the fermentation, optical density increased to 70 (measured at 600 nm). The dissolved oxygen level was controlled by a PI (proportional-integral) controller. Maintaining a glucose concentration lower than 0.5 mM was found to be necessary for avoiding metabolic repression of the pathway converting D-xylose to D-1,2,4-butanetriol.

An improved downstream process was designed, with all individual process steps amenable to industrial scale. This process includes filtration, ion exchange, evaporation and distillation. This process was used to generate material for delivery to the Indian Head Division of the Naval Surface Warfare Center. The purity of the final D-1,2,4-butanetriol was confirmed by HPLC, GC/MS, and proton- and C13 NMR as greater than 99%.

Economic evaluation

An economic model was constructed to assess the costs of producing 15,000 pounds per year of D-1,2,4-butanetriol. As this amount will not justify the capital expense of a new facility, only variable costs of the process are included in the model. Mass- and energy-balances were constructed using the data collected at MBI during the pilot-scale production and isolation of D-1,2,4-butanetriol for delivery to Indian Head. From these balances, the cost model was generated. The costs assigned to raw materials and energy are the current commercial spot prices at the appropriate scale.

The total variable cost calculated for the actual pilot scale production of BT is $74.23 per pound. The costs of glucose, xylose, IPTG, sodium hydroxide, steam, electricity and cooling water are significant contributors. The variable costs can be decreased to $45.23 by improving the activity of the last enzyme in the pathway and by reducing the amount of sodium hydroxide required. The cost can be further lowered to $39.43 by replacing glucose and the IPTG inducer with lactose. The use of glucose *and* xylose generated using MBI's AFEX pretreatment technology and changing the induction system to allow the reintroduction of glucose decreases the cost to $23.78. Finally, doubling the final concentration of BT in the fermentation broth decreases the cost to a low of $18.90 per pound.

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